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Milk Fat Globule Membrane Protein Promotes C_2C_{12} Cell Proliferation through the PI3K/Akt Signaling Pathway

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Abstract

Milk fat globule membrane (MFGM) protein is known to have several health benefits, including an anti-sarcopenia effect; however, its mechanism is unclear. The aim of this study was to investigate the potential mechanism of action of the MFGM protein. The MFGM protein was extracted and separated into 4 fractions, and Fraction 2 (57 % of total MFGM) demonstrated the greatest effect on C_2C_{12} cell proliferation. Milk fat globule-EGF factor 8 (MFG-E8) accounted for 82.35 % of the MFGM protein. The effects of whole Fraction 2 (100 μg/mL, 200 μg/mL and 300 μg/mL) on cell proliferation and morphology were measured. Using qRT-PCR or a Western blot assay, several regulatory factors, e.g., PI3K P85α, p-pI3K p85α (Tyr 508), Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448), were measured in cells incubated with 200 μg/mL of Fraction 2 with or without wortmannin. The results demonstrated that Fraction 2 induced C_2C_{12} cell proliferation in a dose-dependent manner, upregulated the mRNA expression of mTOR and p70S6K, and activated PI3K, Akt, mTOR and P70S6K phosphorylation; however, Fraction 2 inhibited FOXO3a and 4E-BP. The results demonstrate that the MFGM protein, predominantly MFG-E8, promotes cell proliferation through the PI3K/Akt/mTOR signaling pathway. This study elucidated the molecular mechanism of the MFGM protein, primarily MFG-E8, in promoting

C_2C_{12} cell proliferation via the PI3K/Akt/mTOR/P70S6K signal pathway.

Keywords：Milk fat globule membrane; MFG E8; PI3K/Akt/mTOR pathway; C_2C_{12} cell; *Western blot*; sarcopenia.

1. Introduction

Sarcopenia is a disease that is associated with the ageing process in humans. It is associated with a decline in skeletal muscle mass and strength as well as an increase in muscle fatigability [1]. After 30 years of age, lean muscle mass loss takes place at the rate of approximately 1 % every year [2]. Age-related sarcopenia relates to metabolism (anabolism and catabolism of muscle protein). Compared to a younger person, the elderly (aged > 60) produce less muscle protein from the same amount of dietary protein [3], have a slower rate of basal, post-absorptive myofibrillar muscle protein synthesis and have a more negative net protein balance [4]. Medication, hormones, nutrition and resistance training can help treat and prevent sarcopenia. Medication is a significant therapeutic tool; however it has side effects. Recently, research on nutrition regulation of sarcopenia has received broad attention [5, 6].

C_2C_{12} mouse skeletal muscle cells have been widely used as a sarcopenia model to study myoblast differentiation, neuromuscular junction formation and muscle pharmacology [7]. Increased muscle cell apoptosis of C_2C_{12}, in addition to the decline in regenerative potential, contributes to age-associated sarcopenia [8]. Thus, a combined approach targeting both increased regenerative potential and proliferation activity may present a framework for therapeutic intervention of sarcopenia.

Milk fat globule membrane (MFGM) is a mixture of primarily lipids and membrane specific proteins [9]. MFGM has attracted much attention in protection against sarcopenia. Haramizu et al. [10] investigated the effects of habitual exercise plus consumption of MFGM and green tea on aging-related deficits in muscle mass and function in senescence-accelerated P1 mice. The results indicated that exercise plus dietary MFGM can improve muscle function through neuromuscular development. Haramizu et al. [11] evaluated the effects of MFGM consumption on endurance capacity and energy metabolism in BALB/c mice over a 12-week period.

Long-term MFGM intake combined with regular exercise improved endurance capacity, as evidenced by swimming time until fatigue, in a dose-dependent manner.

Phosphatidylinositol 3-kinases (PI3K) are a group of enzymes involved in cellular functions, such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking [12] The PI3K/Akt signaling pathway has been implicated in the regulation of several important cellular processes, including apoptosis survival, proliferation, and metabolism, in skeletal muscle and is activated in response to growth factors, such as insulin-like growth factor-I (IGF-I) [13]. Activation of the PI3K/Akt pathway is sufficient to induce hypertrophy and block skeletal muscle atrophy [14]. Jing et al. [15] reported that PI3K can activate mTOR and downstream target proteins (S6K1, S6, 4E-BP1) as well as increase translation initiation and protein synthesis in skeletal muscles. Xie et al. [16] observed that the PI3K/Akt signaling pathway regulates mTOR/p70S6K activation in C_2C_{12} myoblasts. Activation of PI3K as well as its primary downstream effector, Akt, is necessary for proliferation and differentiation to occur, and PI3K inhibitors, such as wortmannin and LY294002, are able to block myogenesis [17, 18]. Thomas et al. [19] determined the role of LY294002 in the PI3K/Akt signaling pathway in C_2C_{12} myotubes and analyzed the related impact on cell death parameters occurring within this context. Their results indicated that simulated ischemia attenuated PI3K activity is associated with decreased Akt-dependent phosphorylation at the level of mTOR and other target proteins. MFGM has been demonstrated to be an effector in skeletal muscles [10, 11]; however, the molecular mechanism by which the signaling pathway integrates the MFGM protein into skeletal muscle cells has not been previously examined, and whether the MFGM protein activates the PI3K/Akt/mTOR pathway in C_2C_{12} cell proliferation and modulates skeletal muscle protein turnover remains unclear.

Therefore, the primary purpose of the study is to identify the effects of MFGM protein on C_2C_{12} cell proliferation and its signaling pathway. Here, we focused on the pathway involved in MFGM protein modulation of the PI3K/Akt/mTOR signaling pathway.

2 METHODS

DEAE Cellulose DE-52 was purchased from Whatman (Maid- stone, Kent, UK). The Hoechst 33342/PI kit (Solarbio, China) and radio immunoprecipitation assay lysis buffer were purchased from Keygen (Solarbio, China). The BCA protein assay kit was purchased from Thermo Fisher (Pittsburgh, PA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Abcam, Cambridge, Mass., USA). All other chemicals and reagents were purchased from Sigma (Saint-Louis, MO, USA).

2.1 Separation and identification of the MFGM protein

MFGM was prepared as described in our previous study [20, 21] with a slight modification. MFGM (0.2 g) was dissolved in PBS buffer (10 ml, pH 8.0), and the supernatant was applied to a DEAE Cellulose DE-52 (2.6×60 cm), followed by a linear gradient elution of NaCl (0.2 M, 0.5 M and 0.8 M) in the same buffer. Four protein fractions (the elution flow rate was 1 mL/min) were collected and detected at 280 nm by HD-93-1 (Purkinje General Instrument Co. Ltd., Beijing, China). Four fractions were collected after dialysis, concentration and freeze-drying, and their effects on C_2C_{12} cell proliferation were tested. Among the 4 fractions, Fraction 2–MFGM P2–was the largest (57 % of all recovered) and also had the strongest effect on improving cell proliferation (6 times higher than fraction 3; Fractions 1 and 4 both inhibited cell proliferation). Thus, the current study focused on MFGM P2. MFGM P2 was then separated by 1-D gel electrophoresis and the highest density band at 45 kDa was excised. Total MFGM P2 was subjected to the LC-MS/MS assay for further identification of the protein composition based on the method of Churchwell et al. [22].

2.2. Cell Culture and MTT cell activity assay

C_2C_{12} murine skeletal muscle myoblasts were supplied by the Chinese Academy of Agricultural Sciences (Beijing, China). C_2C_{12} cells were grown in Dulbecco's Modified Eagle medium (DMEM, GIBCO) supplemented with 10 % (v/v) fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin, and 100 μg/mL streptomycin

(Invitrogen) in a humidified incubator containing 5 % CO_2 at 37°C.

2.3 MTT cell activity assay

The effect of the MFGM protein on cell proliferation was first measured using the MTT assay [23]. C_2C_{12} cells were inoculated at a density of 5×10^4/ml on 96-well plates and cultured overnight at 37°C. C_2C_{12} cells were then cultured in the same medium with MFGM protein P2 (100 μg/mL, 200 μg/mL and 300 μg/mL) and MFGM (200 μg/mL) (n = 5) for 24 h, 48 h, and 72 h. Next, 20 μl of MTT (5 mg/ml, Sigma) working solution was added to each well and incubated for 4 h at 37°C. After the MTT solution was removed, the formazan that formed inside the cells was dissolved using DMSO. Absorbance was measured at 490 nm using a microplate reader (Model 550, Bio-Rad USA). The cell proliferation rate was calculated using the following equation: (OD_{490} experiment group - OD_{490} control group)/OD_{490} control group. In each group, n = 5.

2.4 Cells cultured with wortmannin and with or without MFGM P2

Wortmannin reagent (Invitrogen, Carlsbad, CA) was introduced to block the PI3K signaling pathway of C_2C_{12} cells. C_2C_{12} cells were inoculated at a density of 5×10^4/ml in 96-well plates and cultured overnight. C_2C_{12} cells were treated with wortmannin at final concentrations of 50 nM/L, 100 nM/L, 200 nM/L, 500 nM/L and 1000 nM/L for 24 h, 48 h, and 72 h. Control group cells were not given wortmannin treatment. Next, C_2C_{12} cell activities were determined by the MTT assay (See section 2.2).

In another experiment, C_2C_{12} cells were inoculated at a density of 5×10^4/mL in 96-well plates and cultured overnight at 37°C. C_2C_{12} cells were then cultured in a medium containing wortmannin (200 nM/L) with 200 μg/mL of either MFGM or MFGM P2 for 24 h, 48 h, and 72 h. Next, the C_2C_{12} cell activity was determined by the MTT assay, as described in section 2.2.

2.5 Confocal scanning laser microscopy (CLSM**) measurements**

The C_2C_{12} cell morphology was measured using the Hoechst 33342/PI kit (Solarbio, China). The method used was as described by Chen et al. [24] with a slight

modification. C_2C_{12} cells were inoculated at a density of 1×10^5/mL in 6-well plates and cultured for 12 h at 37°C. Next, C_2C_{12} cells were treated with 200 nM/L wortmannin for 48 h, followed by addition of 200 μg/mL of the MFGM or MFGM P2 protein to each well and incubation for 48 h. C_2C_{12} cells were then washed with PBS and incubated with 1 mL of cell staining buffer, 5 μL of Hoechst 33342 (excitation 360 nm/emission 465 nm) buffer and 5 μL of PI staining (excitation 488 nm/emission 620 nm) buffer for 30 min at 4 °C in the dark. The percentage of survival and apoptotic cells were then analyzed using CLSM (Leica, Germany).

2.6 Quantitative real-time PCR

Total RNA was isolated from C_2C_{12} cells treated with 200 μg/mL of the MFGM or MFGM P2 protein for 48 h using the RNAprep Pure Cell/Bacteria Kit (TIANGEN, China). For qRT-PCR analysis, cDNA was synthesized by using 3 μg of RNA with the PrimeScript^{TM} II 1st strand cDNA synthesis kit (TaKaRa, China) following the manufacturer's protocol. Quantitative real-time qRT-PCR was carried out on an ABI 7300 Real-Time PCR system (Applied Biosystems) with SYBR Premix Ex Taq^{TM} II (TaKaRa). Relative expression was first quantified using the standard curve method, and the data were normalized to GAPDH mRNA. The primers used in the study are shown in Table 1 [25].

2.7 Western blot analysis

C_2C_{12} cells were treated with 200 μg/mL of either MFGM or MFGM P2 for 48 h, washed twice with PBS buffer and homogenized in lysis buffer. Total protein was extracted and harvested by scraping with a modified radioimmunoprecipitation assay buffer containing 100 nM phenylmethylsulfonyl fluoride for 30 min [26]. Following centrifugation at 10000 rpm for 15 min at 4℃, the supernatant was sonicated. The protein concentration was quantified using a BCA kit (Solarbio, China). Proteins (100 μg) were loaded onto a 1-D SDS gel (10 % polyacrylamide). Next, proteins were transferred onto a nitrocellulose filter membrane (PPLYGEN, China) using a wet electrotransfer system (Bio-Rad, USA）for 4 h at 200 mA. The membranes were blocked with 5 % non-fat dry milk in Tris-buffered saline with Tween-20 (TBST)

buffer for 1 h at room temperature, followed by incubation with primary antibodies (Santa Cruz，USA) for each protein, for 1 h at 37 ℃, or overnight at 4 ℃, according to the requirements. The concentrations of antibodies were as follows: GAPDH (1:500), PI3K (1:500), p-PI3K (1:300), Akt1/2/3 (1:500), p-Akt (Ser 473) (1:800), mTOR (1:300), p-mTOR (1:500), P70S6K (1:800) and p-P70S6K (1:800). The membrane was washed for 5 minutes three times with TBST, followed by incubation with anti-mouse or anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology, Inc.) at 37 ℃ for 1 h. The membrane was washed with TBST twice and with TBS once, 5 min each, and then incubated with alkaline phosphatase until an appropriate signal level was obtained. Protein bands were detected by FluorChem Imaging Systems (Alpha Innotech, Corp., San Leandro, CA, USA).

2.8 Statistical analysis

All experiments, except when otherwise described, were tested and analyzed in triplicate. Analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$) between means. Statistical analysis was performed using a General Linear Model procedure with the SAS 9.1.3 software from SAS Institute, Inc., Cary, NC, USA.

3 Results

The MFGM protein was extracted by an electric cream separator, and four fractions of the MFGM protein were collected by linear gradient elution; the effect of each fraction on cell proliferation was initially assessed. Among the four fractions, the second fraction, MFGM P2, demonstrated the strongest effect–6-fold higher than fraction 3–and both fractions 1 and 4 inhibited cell proliferation. The composition of MFGM protein 2 was then analyzed; the effect of MFGM P2 on C_2C_{12} cell proliferation and its potential for participating in the PI3K/Akt signaling pathway were further investigated. (The work flow is shown in Fig. 1.)

3.1 The composition of MFGM protein Fraction 2, MFGM P2

The components of MFGM P2 were confirmed by SDS-PAGE (Fig. 2A) and LC-MS/MS. The similarity search was based on sequence alignment in Uniprot. The

results demonstrated that there were 62 proteins identified in MFGM P2. The major components of MFGM P2 was lactadherin (MFG-E8), accession NO: tr|Q3T0K7|. Among them, MFG-E8 accounted for 82.35 % of MFGM P2.

3.2 Effect of MFGM and MFGM P2 on cell viability

C_2C_{12} cells were treated with 100 μg/mL, 200 μg/mL or 300 μg/mL MFGM P2 in 96-well plates for 24 h, 48 h and 72 h, and cell proliferation was measured by the MTT assay (Fig. 2B). The results demonstrated that the cell proliferation activity increased with time as well as with concentration, but decreased at 72 h. The proliferation rate reached a maximum (35.8 %) at 200 μg/mL at 48 h. The order of the proliferation rate with different concentrations of MFGM P2 was as follows: 200 μg/mL >100 μg/mL > 200 μg/mL > control > 300 μg/mL. Thus, the concentration of 200 μg/mL of MFGM P2 and incubation time of 48 h were applied for further experimentation.

3.3 Effect of MFGM P2 on the expression of related genes of the PI3K/AKT pathway

The mRNA levels of mTOR, FOXO3a, 4E-BP1 and P70S6K were determined by quantitative real-time PCR experiments (Fig. 3). Compared to the control group, the mTOR and P70S6K mRNA levels in the MFGM group were increased by 10 % and 157 %, respectively; the FOXO3a and 4E-BP1 mRNA levels were reduced by 12 % and 31 %, respectively. Compared to the MFGM group, the mTOR and P70S6K levels in the MFGM P2 group were significantly increased by 109 % and 149 %, respectively, while the FOXO3a and 4E-BP1 mRNA levels were reduced by 5 % and 31 %, respectively.

3.4 Effect of MFGM P2 on signaling molecules involved in protein synthesis and energy regulation of the PI3K /Akt activity

To determine the effects of MFGM P2 on signaling molecules involved in protein synthesis and energy regulation, cells were treated with 200 μg/mL MFGM or MFGM P2 and incubated for 48 h, followed by Western blot analysis. As shown in Fig. 4, PI3K P85α, p-pI3K p85α (Tyr 508), Akt, p-Akt (Ser 473), mTOR and p-mTOR

(Ser 2448) were all observed to be constitutively expressed during C_2C_{12} cell proliferation. Compared to the MFGM group or control group, the endogenous levels of both PI3K p85 and p-pI3K p85α (Tyr508) subunits were increased. Among them, total mTOR expression was not altered significantly, but the p-mTOR levels were significantly increased compared to both the control and MFGM groups. These results demonstrate that both MFGM and MFGM P2 not only increase the total PI3K, AKT mTOR and p70S6K protein levels but also increase the phosphorylated PI3K, AKT, mTOR and p70S6K protein levels. Therefore, both MFGM and, especially, MFGM P2 significantly increased C_2C_{12} cell proliferation via activation of the PI3K/Akt pathway.

3.5 Effect of MFGM or MFGM P2 with wortmannin on cell viability

To further investigate the role of PI3K/AKT signaling on the effect of MFGM P2 on C_2C_{12} cell proliferation, wortmannin–an inhibitor of PI3K–was used to enhance the sensitization of C_2C_{12} cells to investigate whether C_2C_{12} cell proliferation, which his improved by MFGM P2, predominantly MFG-E8, activated the PI3K signaling pathway.

3.5.1 Effect of wortmannin on the growth of C_2C_{12} cells

To determine whether inhibition of PI3K activity by wortmannin affects cell growth, C_2C_{12} cells were treated as described above, and cell growth was analyzed by the MTT assay. The effects of wortmannin on the growth of C_2C_{12} cells are shown in Fig. 5. Cell growth was inhibited with each wortmannin treatment (50 nM/L, 100 nM/L, 200 nM/L, 500 nM/L or 1000 nM/L for 24 h, 48 h and 72 h) in a time- and concentration-dependent manner. An inhibition rate of 43.8 % was achieved at a concentration of 1000 nM/mL during 48 h. The order of inhibition rate of wortmannin, from high to low, was as follows: 1000 nM/L > 500 nM/L > 200 nM/L > 100 nM/L > 50 nM/L. However, considering the cell growth activity and dose-inhibition rate of the drug, the concentration of 200 nM/L wortmannin and 48 h incubation time were applied for further experiments.

3.5.2 Effect of MFGM P2 on the proliferation of C_2C_{12} cells

To investigate the effect of the MFGM protein on the PI3K signaling pathway, the effects of wortmannin with 200 μg/mL of MFGM or MFGM P2 on the growth of C_2C_{12} cells for 24 h, 48 h and 72 h were examined. The results (Fig. 5 B) demonstrated that cell proliferation activity increased with time as well as with concentration, but decreased at 72 h. The proliferation rate reached a maximum (22.8 %) with 200 μg/mL for 48 h. The order of proliferation rate was as follows: MFGM P2 > MFGM > control. Thus, wortmannin with MFGM P2 and an incubation time of 48 h were applied for further experiments.

3.5.3 Effect of wortmannin and MFGM P2 on C_2C_{12} cell viability

The results of CLSM demonstrated that the cell number in the normal group was higher than that in the wortmannin group, and the highest cell numbers were seen in the MFGM P2 group. The order of the magnitude of cell numbers from high to low was as follows: MFGM P2 group (55.6 %) > MFGM (22.2 %) > Control, which was calculated by (cell number in assessed group-control group)/control group (Fig. 6).

3.6 Activation of the PI3K/AKT signaling pathway by MFGM P2

PI3K activation is typically indispensable for cell proliferation, PI3K plays a central role in cell signaling and leads to cell proliferation, survival, motility, secretion, and specialized cell responses, such as the respiratory burst of granulocytes [27]. To confirm whether the MFGM protein affects the PI3K/AKT signaling pathway in C_2C_{12} cells, the PI3K protein and phosphorylation levels were detected using Western blot analyses. Compared to the control and MFGM groups, MFGM P2 promoted the phosphorylation of PI3K in C_2C_{12} cells (Fig. 7 A).

3.6.1 Effect of MFGE8 and wortmannin on Akt and phospho-Akt serine 473

PI3K and Serine 473-phosphorylation of Akt was increased significantly ($p<0.03$) in cells cultured with MFGM P2 compared to both the control and MFGM groups. The extent of alterations in the AKT levels observed after MFGM P2 treatment reflected the previous findings for PI3K and serine 473 (Fig. 7B). Compared to the group with only MFGM P2, approximately 23.3 % of phosphorylation of Akt at the serine 473 position was decreased in cells cultured with both wortmannin and MFGM

P2. Analogous results were seen in other reports where wortmannin or another inhibitor (LY294002) was added to the medium [19, 27, 28].

3.6.2 Effect of MFGM P2 and wortmannin on mTOR and p-mTOR Ser2448

Akt phosphorylates mTOR directly at the serine 2448 motif [29]. Compared to the control and MFGM groups, a significantly higher Akt-dependent phosphorylation of mTOR was observed after treatment with MFGM P2 at 48 h. Inhibition of the PI3K pathway with wortmannin during acute MFGM P2 treatment also led to statistically significant increase in Akt dependent mTOR phosphorylation (Fig. 7C).

3.6.3 Increased phosphorylation of P70S6K in response to Akt knockdown

Akt is the principal molecule involved in PI3K-mediated mTOR activation, which is an important parameter that was used to identify the levels of phosphorylated P70S6K. In this study, to determine whether activation of P70S6K resulted from PI3K-induced activation of mTOR, wortmannin was introduced. The results demonstrated that P70S6K phosphorylation and activation were prevented by wortmannin. Compared to the control and MFGM groups, phosphorylation of P70S6K was significantly higher after treatment with MFGM P2 at 48 h（Fig. 7D).

In sum, these results indicate that MFGM P2 promotes expression of the PI3K/Akt/mTOR signaling pathway-related total proteins as well as phosphorylated proteins, and the ratio of phosphorylated protein to total protein was increased.

Discussion

The C_2C_{12} murine myogenic cell line is a popular model for studying myogenesis *in vitro* [30]. Previous studies [10, 11] demonstrated that MFGM improves cell proliferation. As MFGM contains a variety of components, it is important to investigate which individual component is responsible for the improvement of cell proliferation and what mechanisms underlie this role.

To achieve these objectives, first, cellulose DEAE-52 was used to purify MFGM proteins, which were further separated into 4 fractions; the effect of each individual fractions on C_2C_{12} cell proliferation was tested. Among the four fractions, fraction 2, MFGM P2, showed the best performance. The composition of this protein was further

identified by LC-MS/MS. Among the 62 proteins identified in fraction 2, MFG-E8 composed 82.35 % of the total protein. MFG-E8 is also known as lactadherin, a protein encoded by the MFG-E8 gene in humans. MFG-E8 is a major glycoprotein of the milk fat globule, a protein and triglyceride-rich membrane-bound vesicle secreted from the mammary epithelium during milk production [31]. Previous_studies have demonstrated that MFGM P2 can promote C_2C_{12} cell proliferation based on the results of flow cytometry, laser confocal microscopy and transmission electron microscopy analyses [32]. In this study, the anti-sarcopenia effect of MFGM P2 was analyzed. Because MFG-E8 comprised 82.35 % of the total protein in MFGM P2, the effect of MFGM P2 was considered to be a potential effect of MFG-E8, even though further research is required for verification of this hypothesis.

The results from the MTT assay suggested that all three concentrations of MFGM P2 improve cell proliferation, but the highest proliferation rate was in cells cultured with 200 μg/mL. This dosage is a good reference for future clinical trials. The results from confocal scanning laser microscopy demonstrated that the C_2C_{12} cell density was higher in the MFGM P2 group than in the MFGM group. Therefore, MFGM P2, predominantly MFG-E8, was more beneficial for improving cell proliferation.

The PI3K/Akt signaling pathway plays a central role in diverse cellular function, including proliferation, apoptosis, cell survival and metabolism [33, 34]. Overexpression of Akt has been observed to promote cell proliferation and prevent apoptosis in several cell types, resulting in a resistance to or delay of cell death. Few studies have demonstrated the effect of MFG-E8 on the PI3K/Akt signaling pathway [35, 36]. Neutzneri et al.[36] have reported that MFG-E8 regulates colorectal cancer cell migration, invasion and epithelial-to-mesenchymal transition through activation of the PI3K/Akt signaling pathway, which inhibits cell growth and promotes cell apoptosis. As shown in Fig. 3, the results indicate that MFG-E8 may regulate C_2C_{12} cell proliferation through activation of the PI3K/Akt signaling pathway via an increase in mTOR and P70S6K protein expression and inhibition of FOXO3a and

4E-BP1 protein expression (Fig. 3). Furthermore, the effect of MFG-E8 on the PI3K/Akt signaling pathway-related total protein and phosphorylation protein was investigated by western blot analysis.

Akt promotes cell proliferation through phosphorylation of several downstream effectors and the regulation of various transcription factors [19]. The results demonstrated that expression of the target genes FOXO3a and 4E-BP was inhibited by inhibition of their transcriptional activity, whereas the transcriptional activities of mTOR and P70S6K were enhanced. However, compared to the inhibition of the transcriptional activity of FOXO3a and 4E-BP, the promotion of transcriptional activity of mTOR and P70S6K was significant. Thus, the elevated PI3K/Akt activity in C_2C_{12} cell proliferation may be due to an increase in MFG-E8. Which in turn is tightly regulated by mTOR, a key controller of protein synthesis in the cell.

The above conclusion was confirmed by the results of the western blot assay (Fig. 4), which demonstrated that MFGM P2 regulated transcription and expression of the PI3K P85α, p-pI3K p85α (Tyr 508), Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448) to affect cell growth. Previous studies demonstrated that the role of MFG-E8 in cell growth, invasion, and metastasis promoted resistance to apoptosis, epithelial-to-mesenchymal transition, and angiogenesis through activation of the PI3K/Akt signaling pathway [35, 37]. However, there have been no reports regarding the effect of MFG-E8 on C_2C1_2 cell growth. Promotion of cell proliferation by MFGM P2 might be related to the effect of MFG-E8 on the PI3K/Akt/mTOR signaling pathway. The western blotting results for several proteins linked to the PI3K/Akt/mTOR signaling pathway demonstrated that MFGM P2 plays a vital role in cell proliferation via the PI3K pathway (Fig. 5). To further confirm that MFG-E8 promotes C_2C_{12} cell proliferation via the PI3K/Akt signaling pathway, the effect of MFG-E8 on PI3K/Akt signaling pathway-related total protein and phosphorylated protein expression was examined. Wortmannin, a selective inhibitor of the PI3K pathway, was introduced. The results demonstrated that there was a gradual decrease in the MTT reductive capacity when C_2C_{12} cells were incubated with increasing

concentrations of wortmannin for 24 h, 48 h and 72 h (Fig. 2 B). There was a significant decrease in MTT reductive capacity, which was only achieved when using a high concentration of wortmannin combined with MFGM P2. The promotion of skeletal myoblast cell proliferation by MFG-E8 was inhibited by the PI3K inhibitor wortmannin.

Akt (also named protein kinase B) is a serine/threonine kinase, operates downstream of PI3K, and has a key responsibility in regulating signaling pathways concerning cell survival [19, 38]. Akt activation prevents muscle atrophy including sarcopenia [39]. Phosphorylation of Akt at both its serine 473 and threonine 308 motifs is necessary for its complete activation [40]. Inhibition of Akt activation with wortmannin, also blocks activation of PI3K, depressing the expression of p-Akt Ser473 [41]. In this study, compared to the normal group, PI3K, Akt，mTOR, and P70S6K phosphorylation were reduced when C_2C_{12} cells were cultured with both MGFM P2 and wortmannin, which further indicated that MFG-E8 targets Akt phosphorylation, which is regulated upstream by PI3K in the model. Wortmannin can not only bind to class I PI3K as well as other PI3K-related kinases but can also bind to other novel targets that are seemingly unrelated to the PI3K family [19, 42]. It is noteworthy that the decrease in p-Akt was comparatively small given the large decrease in the PI3K activity [19, 43].

mTOR is a key enzyme in the PI3K/AKT signaling pathway, the activity of which is firmly regulated by cellular nutrient availability. Depletion of amino acids diminishes mTOR-dependent IGF-II expression in C_2C_{12} myogenesis [44]. MFG-E8 increased intracellular mTOR phosphorylation. mTOR is negatively regulated by a heterodimer made up of the TSC (tuberous sclerosis complex) proteins: TSC1 and TSC2. TSC2 has been defined as a target for Akt phosphorylation *in vivo*/*vitro* [19, 45]. Indeed, a decrease in Akt-dependent stimulation of phospho-TSC2 and phospho-mTOR was observed during MFG-E8. Supplementation with a PI3K inhibitor confirmed the role for PI3K control at these motifs. Jozwiak et al.[45] reported that mTOR regulates synthesis of both proteins, via phosphorylation and

activation of p70S6 kinase (p70S6K), as well as by phosphorylation and release of the inhibitory effect of initiation factor 4E-BP1 and protein degradation via transcription factors of the Foxo family. Phosphorylation of both p70S6K and 4E-BP1 leads to the activation of a pathway that promotes protein synthesis and translation initiation. Therefore, supplementation with a PI3K inhibitor confirmed the role for PI3K control at these motifs.

In summary, the MFGM protein promotes C_2C_{12} cell proliferation through activation of the PI3K/Akt/mTOR signaling pathway (Fig. 8), promotes synthesis of the regulatory and catalytic subunit of PI3K, and causes PI3K p85 subunit translation as well as an increase in its relative capacity for catalyzed production of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate). PDK1(3-phosphoinositide dependent protein kinase-1) binding to the cell membrane by plekstrin homology domain activates PI3K. Phosphorylation of Akt ser473 was increased by PDK2, and Akt was activated. The downstream target TSC2 was phosphorylated by the GTP protein kinase and its activity was inhibited, resulting in activation of mTOR [46]. These results were obtained in a C_2C_{12} cell model; however, one cell line may not be enough to certify the mechanism of the MFGM protein on C_2C_{12} cell proliferation. Furthermore, the effects of MFG-E8 on skeletal muscle growth and expression of the PI3K/Akt pathway-related proteins will be validated in rats via in vivo experiments. In addition to the pathway that we explored, several other unknown factors might be involved in cell proliferation.

Conclusion

This study provides a novel insight into the positive effect of the MFGM protein, especially MFG-E8, on the improvement of sarcopenia and its potential mechanisms underlying the PI3K/Akt signaling pathway in C_2C_{12} cells, a model of sarcopenia. Experiments using an animal model are being conducted for further confirmation.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Table

Table 1. Primer sequences

Primer	Forward primer	Reverse primer
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
p70s6k	GGGAAGGCTTTGCAGTTTAC	TCCAGTCCCTCACGAACAAA
4E-BP1	CCAGCAGCCCGGAAGATAA	GGTCCCTTAAATGTCCATCTCA
mTOR	CTGGGACTCAAATGTGTGCAGTTC	GAACAATAGGGTGAATGATCCGGG
FOXO3a	TCGCGCACCAATTCCAAC	TCGCTGTGGCTGAGTGAGTC

Figure

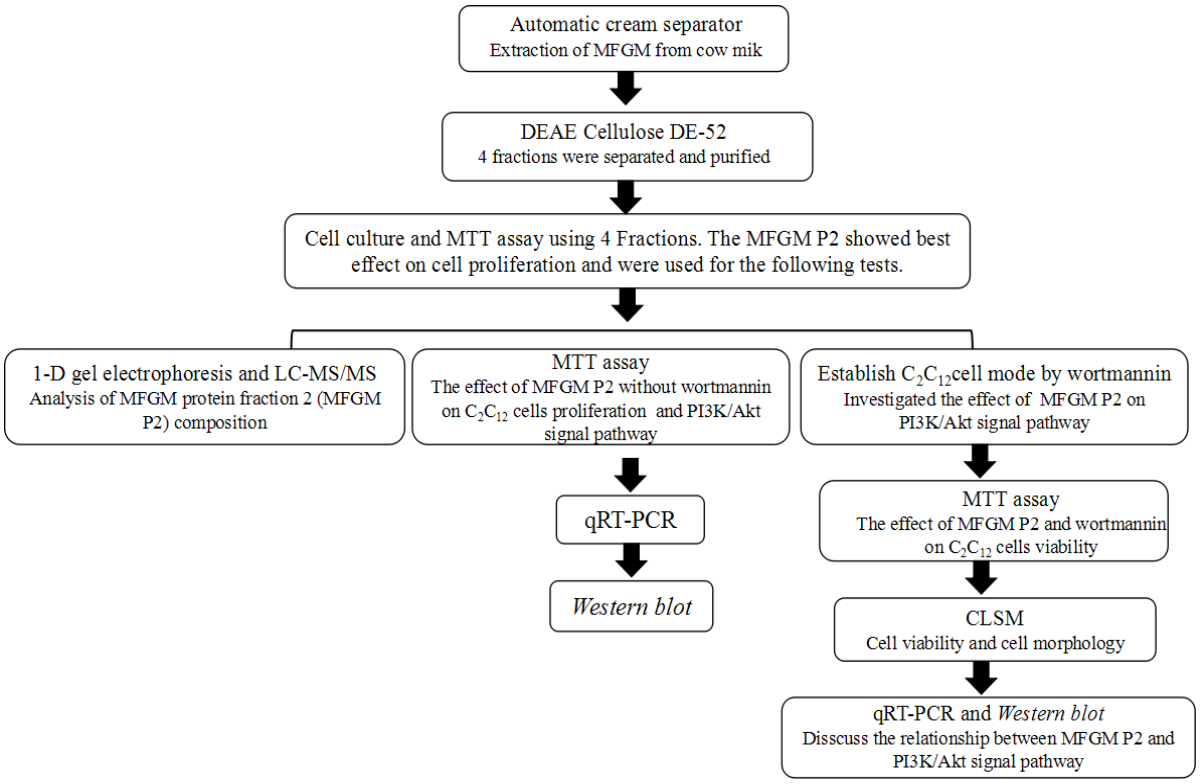


Figure. 1 The flow chart of this study

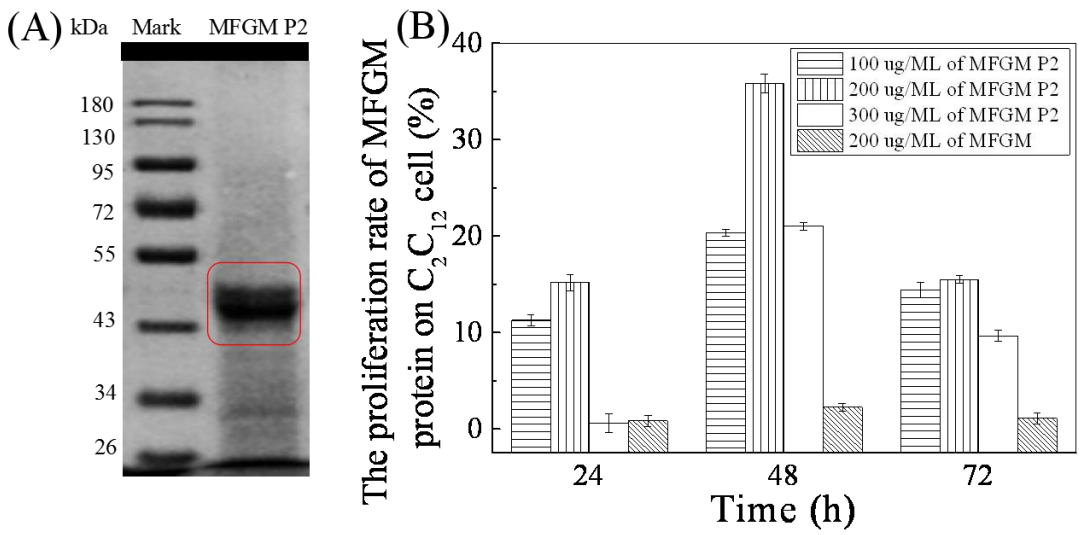


Figure. 2 SDS-PAGE analysis of the MFGM P2 (A), and the effect of MFGM P2 on C_2C_{12} growth(B).

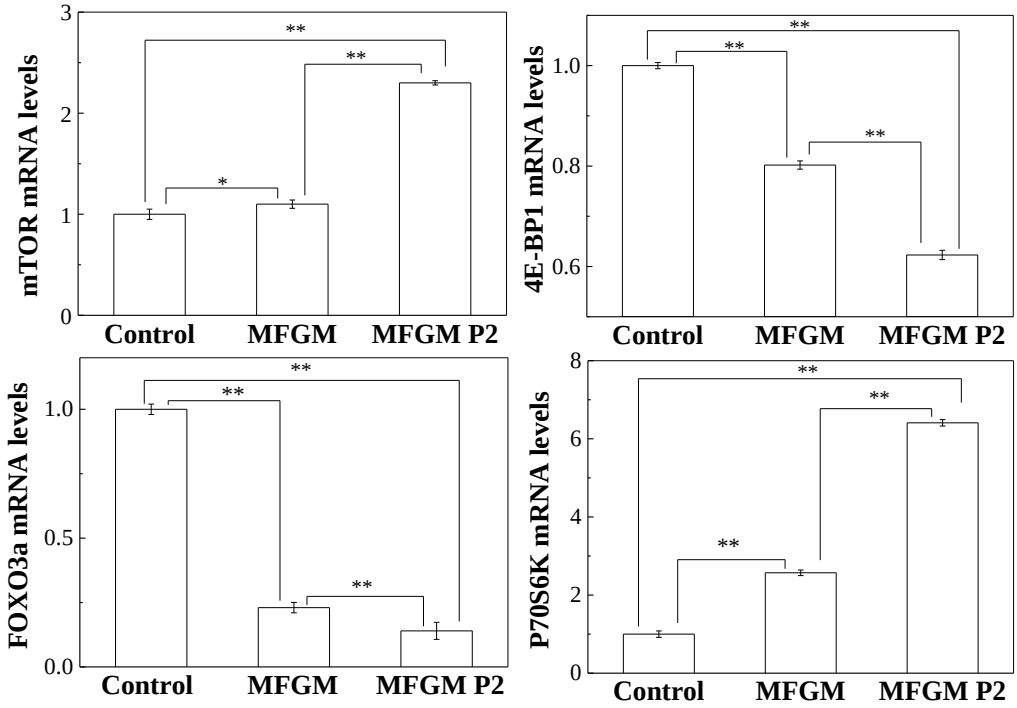


Figure. 3 The effect of MFGM P2 on mRNA levels of mTOR, FOXO3a, 4E-BP1 and P70S6K at 48 h; The relative mRNA levels were calculated using the $\Delta\Delta C_t$ methods, $\Delta\Delta$Ct=target group (CT of target genes -CT of CAPDH)-control group (CT of target genes -CT of GAPDH), and data were expressed as a fold change (n=4). The results are expressed as mean ± SEM, *p<0.05, **p<0.01 vs. Control and MFGM (n=3).

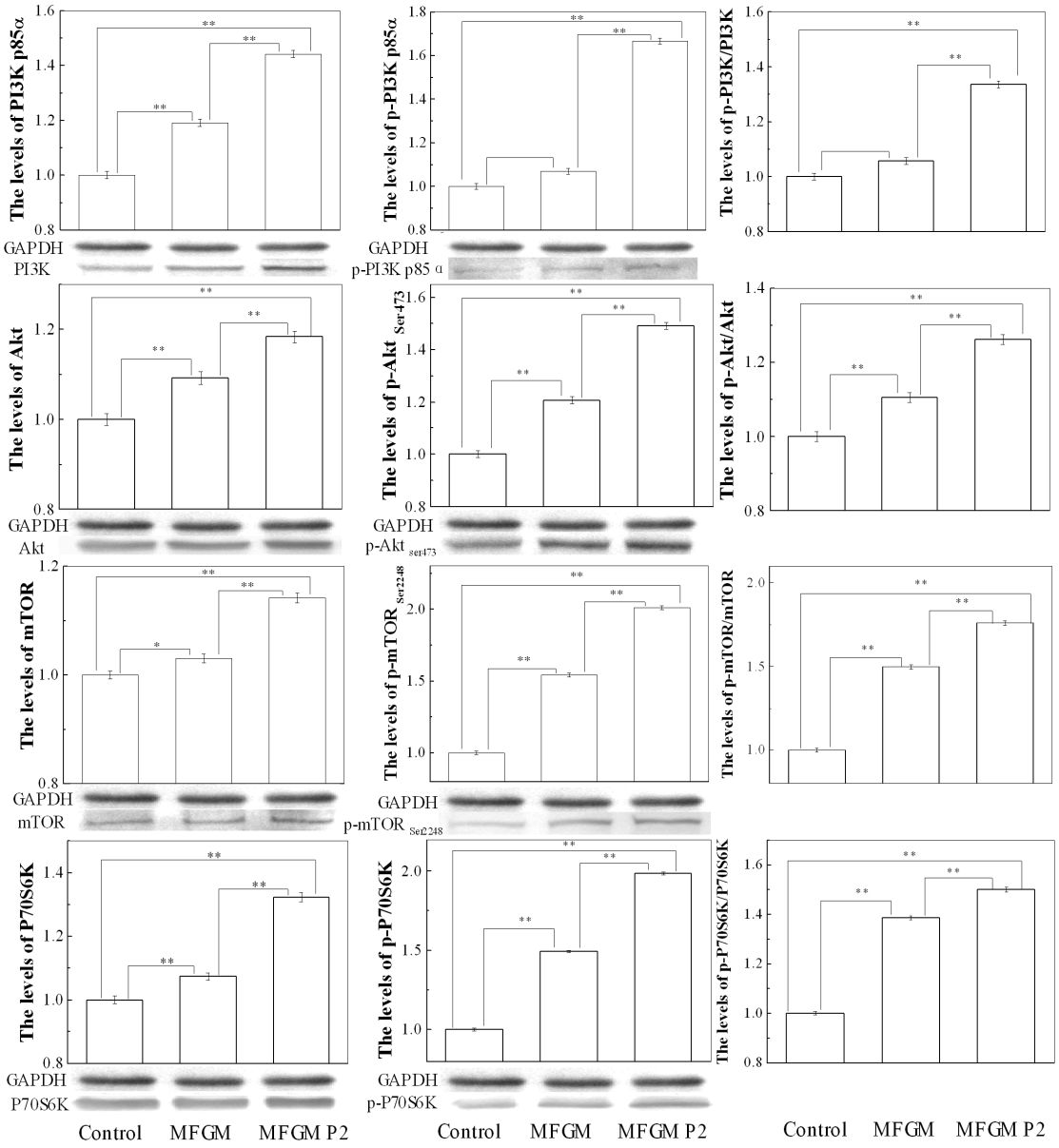
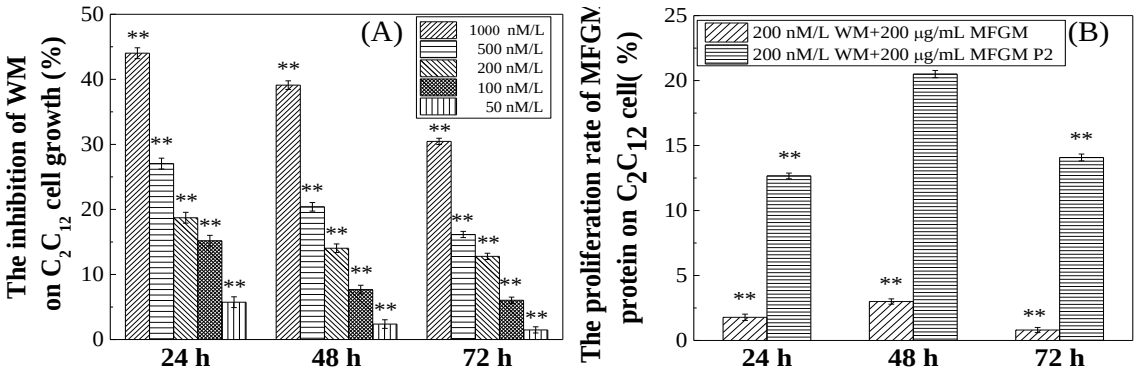


Figure. 4 The effect of MFGM or MFGM P2 on PI3K, p-PI3K, Akt, p-Akt Ser 473, p-mTOR Ser 2448, p70s6k and p-p70s6k expression at 48h. The results are expressed as mean ± SEM, *p<0.05, **p<0.01 vs. Control or MFGM (n=3).



Figrue. 5 The effect of WM or MFGM P2 on cell viability. A) The effect of WM on C_2C_{12} cell growth, the cell inhibition rate was calculated as in the following equation: (OD490 experiment group - OD490 control group)/OD490 control group. In each group, n = 5.*p<0.05, **p<0.01 vs. Control; B) The effect of wortmannin (200 nM/L) and MFGM P2 (200 μg/mL) on cell growth,* p<0.05, **p<0.01 vs. Control or MFGM (n=4).

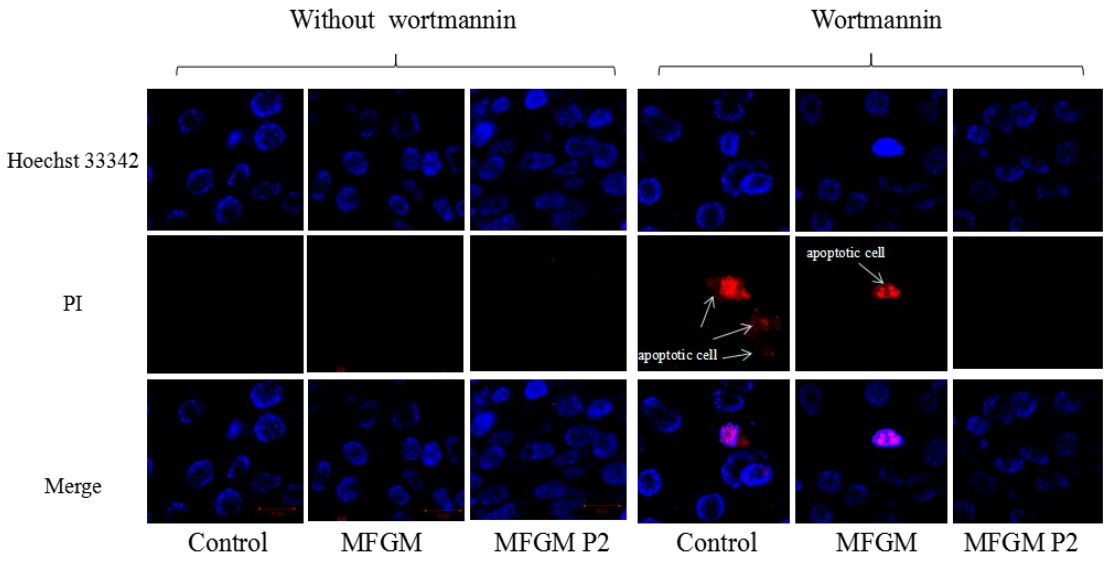


Figure. 6 The effect of MFGM P2 on cell morphology by CLSM analysis. Wortannin can inhibit cell grow, even increase apoptosis (arrows), which can be blocked by MFGM P2.

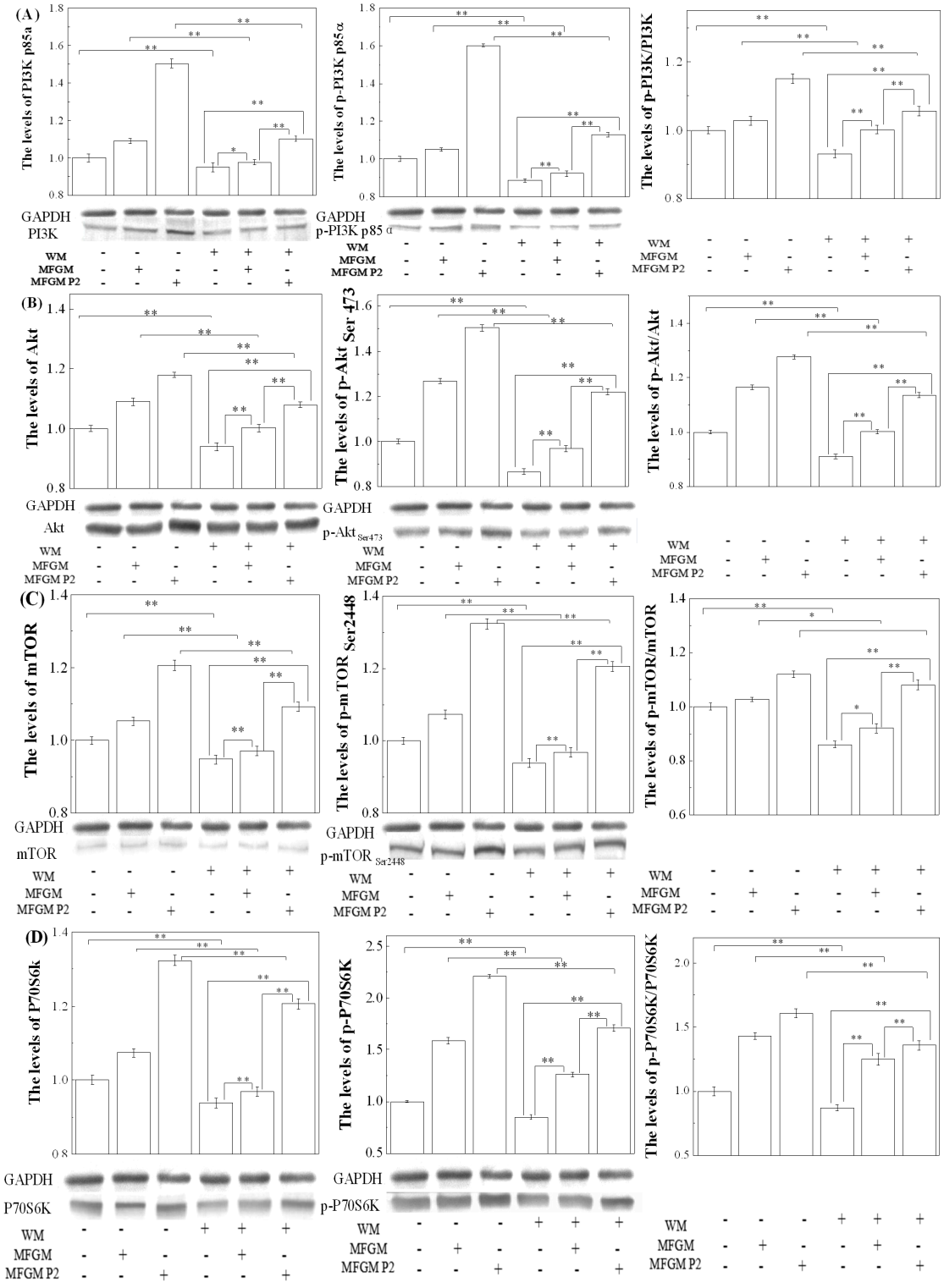


Figure. 7 The effect of individual or combined with MFGM, MFGM P2 and wortmannin on the expression of total and phosphorylated PI3K p85α (Tyr 508) (A); Akt. (Ser473) (B); mTOR (C), p70S6K (D). Data are presented as mean ± S.E.M, **p<0.01, *p<0.05 (n=3).

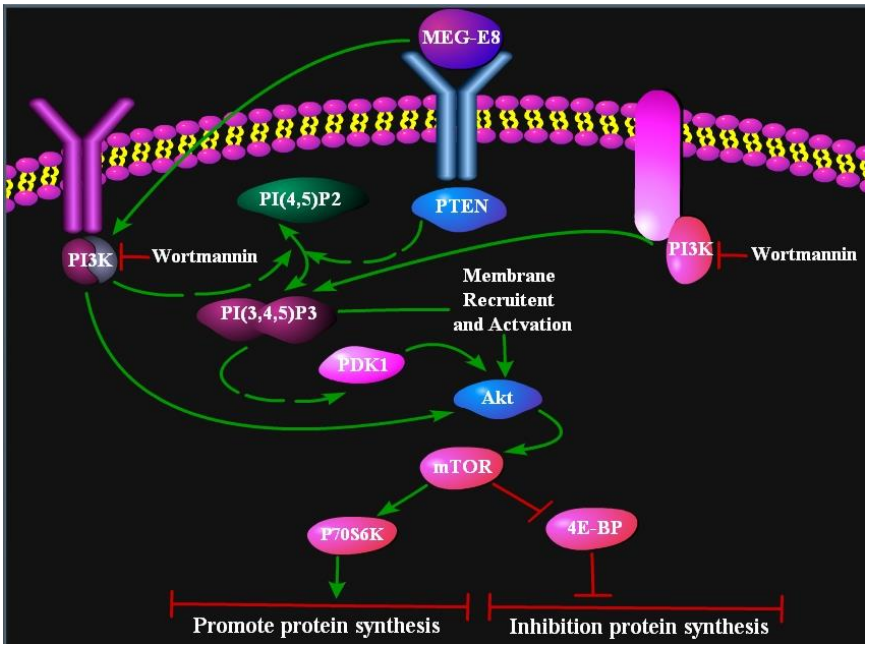


Figure. 8 The role of MFG-E8 in C_2C_{12} cell proliferation via PI3K/Akt signal pathway. Based on the results, we proposed that the mechanism of MFG-E8 on C_2C_{12} cell proliferation might be described as follows: MFG-E8 promotes the regulatory and catalytic subunit of PI3K synthesis, PI3K p85 subunit translation was induced and further increased relative capacity for catalyzed production of PIP3. Then PDK1 binding to the cell membrane by the plekstrin homology domain, and with subsequent PI3K activation, then phosphorylation of Akt ser473 was increased by PDK2, and Akt was activation completely. The downstream targets TSC2 was phosphorylated and the GTP protein kinase activity was inhibed, mTOR compound was activated, mTOR regulation both protein synthesis, via phosphorylating and activating p70S6K, as well as phosphorylating and releasing the inhibitory effect of initiation factor 4E-BP1 and protein degradation. The diagram of the mechanism of MFG-E8 on cell proliferation was modified from Jeong, Gao and Huang et al [47-49].

Highlights

•The main protein in MFGM protein fraction 2 was milk fat globule-EGF factor 8 (MFG-E8), it was identified and quantified by LC-MS/MS.

•The effect of MFG-E8 on C_2C_{12} cell growth was analysed by MTT assay, CSLM, qRT-PCR and *Western blot*.

•The mechanism of C_2C_{12} cell proliferation was analysed by qRT-PCR and *Western blot*.